Indian Journal of Ecology (2020) 47 Special Issue (12): 245-251

Manuscript Number: 483
NAAS Rating: 4.96

Effect of Types Coating on Survival *of Lactobacillus casei* and Stability of Simulated Gastrointestinal Conditions

Read R. Al-Hatim, Ali K. AL-Rikabi and Amal K. Ghadban

Department of Agricultural Food Science, College of Agriculture, University of Basrah, Iraq
E-mail: raqadraheem@yahoo.com

Abstract: The variation in the efficiency of the binding process between coating types during configure the simulated gastric fluids (SGF), the bio probiotic cells coated with a double-layer coating resulted in highest stability to the pepsin enzyme with a survival rate of 94.41%, while free cells recorded the lowest survival rate of 68.15% and the highest reduction rate was 2.92. The bio probiotic cells coated with double layer coating the highest stability towards pH (without enzyme) with a survival rate of 76.4%, whereas in the free cells survival rate was 64.5 and the highest reduction rate was 3.25, and as for the results in configure the simulated intestinal fluid (SIF)/ The encapsulated uncoating beads was recorded the cells release percentage towards neutral medium was 45%, whereas in the bio probiotic cells with double layer coating was lowest (14%) at 30 minutes. The swelling ratio results of encapsulated and double layer coating beads in the stomach and intestines it was lowest under the effect of pepsin and pancreatic enzymes.

Keyword: Microencapsulation, Proteins, Extrusion method, Coating type, Chitosan, Simulated gastric fluid (SGF), Simulated intestinal fluid (SIF)

The micro-packaging is in the form of liquid drops, and it is a process of retention the sensitive compounds, bioactive compounds, bacteria cells, vitamins, decomposed lactose, essential oils, omega-3 oils and plant extracts and pigment in an environment where bioactive compounds cannot be released. It is a thin layer protects the material from degradation and interactions that make them less effective, and it works to stabile the bio probiotics to give them additional protection. The effect of microencapsulation technique on the preservation of bacteria cell numbers and their ability to survive in sufficient numbers during the manufacturing stages and prolonging the shelf-life, and it does not effect on the sensory properties of flavor, taste, color, resistance to bile salts, acidity, enzymes and oxygen risks (Feucht and Kwak 2013). Milk proteins are one of the basic components that fall within the functional foods, which are a group of foods contain on some of the food ingredients that have a healthy effect. Milk proteins are one of the materials used for microencapsulation of therapeutic bacteria and characterized by protein hydrogel. The important qualities of these proteins: 1- Proteins have the ability to form gel, emulsion, and foam and stabilizers material. 2- Whey proteins are characterized as globular proteins. 3- There are a large number of dairy products, which characterized by different compositions depending on the industry method and variation in the characteristics and functions, these compositions include whole milk protein (Lazidis et al 2016, Siamand et al 2014, Tripathi and Giri 2014, Vivek 2013). To protect the active compounds and the bacteria through its exposure to the manufacturing conditions and storage, which leading to its degradation by means of temperature, moisture and oxygen, as well as its decomposition in the digestive tract, then it's exposed to stomach acidity and bile salts and the presence of intestinal enzymes, it must develop the micro-packaging techniques (Kailasapathy 2006). The selection of coating method depends on the physical and mechanical properties of the polymer, the coating materials applications and releasing the location of active compounds and the material cost. Consideration should be given to the bio probiotic properties to be coated and coating type and the polymer composition (Burgain et al 2011, Parra-Huertas 2010). The microencapsulation technique in the extrusion method that called the droplet system is most common in produce micro beads for colloids in coating and it has unique characteristics (Feucht and Kwak 2013). The extrusion method is based on mixing bio probiotic with polymer in the solidification solution and syringe pressing leads to forming micro beads. The extrusion method can be done in two steps. The first step is mixing the bio probiotic with polysaccharides and forming the beads in the solidification solution. The second step is coating with polymer; this step is called the Mono layer coating (Anal and Singh 2007, Amine et al 2014). Spray drying technology is a new method currently developed. It could be an alternative to extrusion and emulsion technology. This method is preferred when using the microencapsulation of effective compounds, including enzymes, oils and phenolic compounds (Nazzar et al 2012). Recent years have

seen a broad trend in using encapsulation and coated beads through the use of coating materials, but it is differed in the method of coating and the concentration of the used materials by the variation of material charge with the layer coating of encapsulated beads. For the successful coating process, the types of coating can be classified according to the method used, which includes the Mono or single layer coating; Duoble layer coating and Complex layers coating (Shi et al 2013, Li et al 2011, Mokarram et al 2009). Annan et al (2008) encapsulated probiotic cells in alginate coated gelatin microspheres the encapsulation yield (Ey) of 41-43, the measurement of the efficacy of retention and survival of viable cells during the microencapsulation procedure, conducted a comparative study between the numbers of coated and free cells of bio probiotic through the coating with gelatin and configure the (SGF) and (SIF) at the pH 2and pH 7 for 4 hours, and it was observed that the viable cunts log (cfu/ml)of coated bacteria cells ranged between 7.6-7.4, while the viable cunts log (cfu/ml) of free bacteria cells ranged from 6.7 to 6.4. Also showed that encapsulation to provide better protection for the bio probiotic during it passes through the stomach, and its resist the acidic medium and enzymes. As (Mokarram et al 2009) compared through using different encapsulation stages for multi stage alginate, where used beads loaded with bacteria without coating called uncoating beads, and coating beads with the single layer called mono layer and coating with two layers called double layer, and the free cells and make it under the (SGF) and (SIF) at two PH 1.5 and 7.5 for two hours, respectively, where it was observed that the logarithmic number of bacteria, *L. acidophitus* was 3.4 logarithm / ml, while the logarithmic number of bacteria *L. rhamnosus* is 4.1 log (cfu/ml) in the double-layer coating, and the viable counts of *L. acidophitus* bacteria is 7.3 log (cfu/ml), whereas the viable cunts (cfu/ml) of *L .rhamnosus* bacteria is 2.2 (cfu/ml) in mono layer coating, while in the free cells its viable cunts (cfu/ml) from 2.0 to 2.2 respectively at pH 7.5.

MATERIAL AND METHODS

Preparation of culture: *Lactobacillus casei* DSM2001 (University of Tehran college of Agriculture and Natural Resources Daneshkade Karaj, Iran) were inoculated transferred into 9ml MRS (Man Rogosa Sharpe) broth Hi-Media/ India *,Lactobacillus casei* was activated in MRS-Broth liquid medium at 37 °C for 18 hours in incubator supplied with 5% CO_2 and then matched with standard McFarland tubes prepared by (Garvy et al 1977).

Microencapsulation Method

Preparation of encapsulated uncoating beads: The recommended method (Gunasekaran et al 2013) with some modifications was used. The buffalo whey at concentration of 15% was mixed with deionized water using magnetic stirrer for two hours, then it was preserved in the refrigerator for 16 hours. The heat treatment was performed at 85°C for 15 min in a water bath and the pH was adjusted to 6.8, then the solution was cooled at 22°C and mixed with the bacteria in a volume of 10 ml. The mixer for homogeneity in a numbers of 15 x 10^8. The extrusion method was used by a syringe on a cooled calcium chloride solution 0.2 M which was placed on a magnetic stirrer at speed 100 rpm. The solution formation of the beads was rinsed with calcium chloride solution for 30 minutes, then washed with sterile physiological solution for the purpose of disposal the calcium chloride This was filtered, preserved in sterile distilled water and stored in tubes containing sterile 0.1% peptone solution at a temperature of 4°C.

Preparation of single coating beads: The method of Krasaekoopt et al (2004) with some modifications was used. The encapsulated and coated beads were removed from the alginate solution and poured into a flask with funnel supplied with a Whatman No.1 filter paper for the purpose of disposal the alginate solution and then placed a layer of perforated thermal paper. This process took 30 minutes, and followed by the same steps as above

Preparation of double coating beads: The method proposed by Krasaekoopt et al (2004) was used, the coated beads were removed from the alginate solution and poured into a flask with funnel supplied with a Whatman No.1 filter paper for the purpose of disposal the alginate solution, and then placed a layer of perforated thermal paper, then followed by the same steps as described in paragraph 1.

Bacterial enumeration of free cell, encapsulated, coated cells: The numbers of free cells, releasing encapsulated cells and bacteria numbers was done as per standard method (Mathews 2007). 1 g of encapsulated and coated cells was transferred to the test tubes containing 9 ml of sterile solution of cell releasing and then transferred to a vibrator incubator at 130rpm at a temperature of 37°C for 15 min, then 1 ml of cells releasing from beads solution was transferred after incubation to a tube containing 9 ml of peptone solution, serial dilution was performed. The 100µL were withdrawn from each dilution and placed in a petri dish, then poured MRS agar with L-cysteine HCl. a Then incubated in anaerobic conditions at 37°C for 72 hr, while the active free cells, 1 ml was transferred to a tube containing 9 ml of peptone solution and then a serial dilution was performed, then it was cultivated and incubated under the same conditions.

Encapsulation yield (EY): To determine the effects of concentration and type of coating materials, the yield of

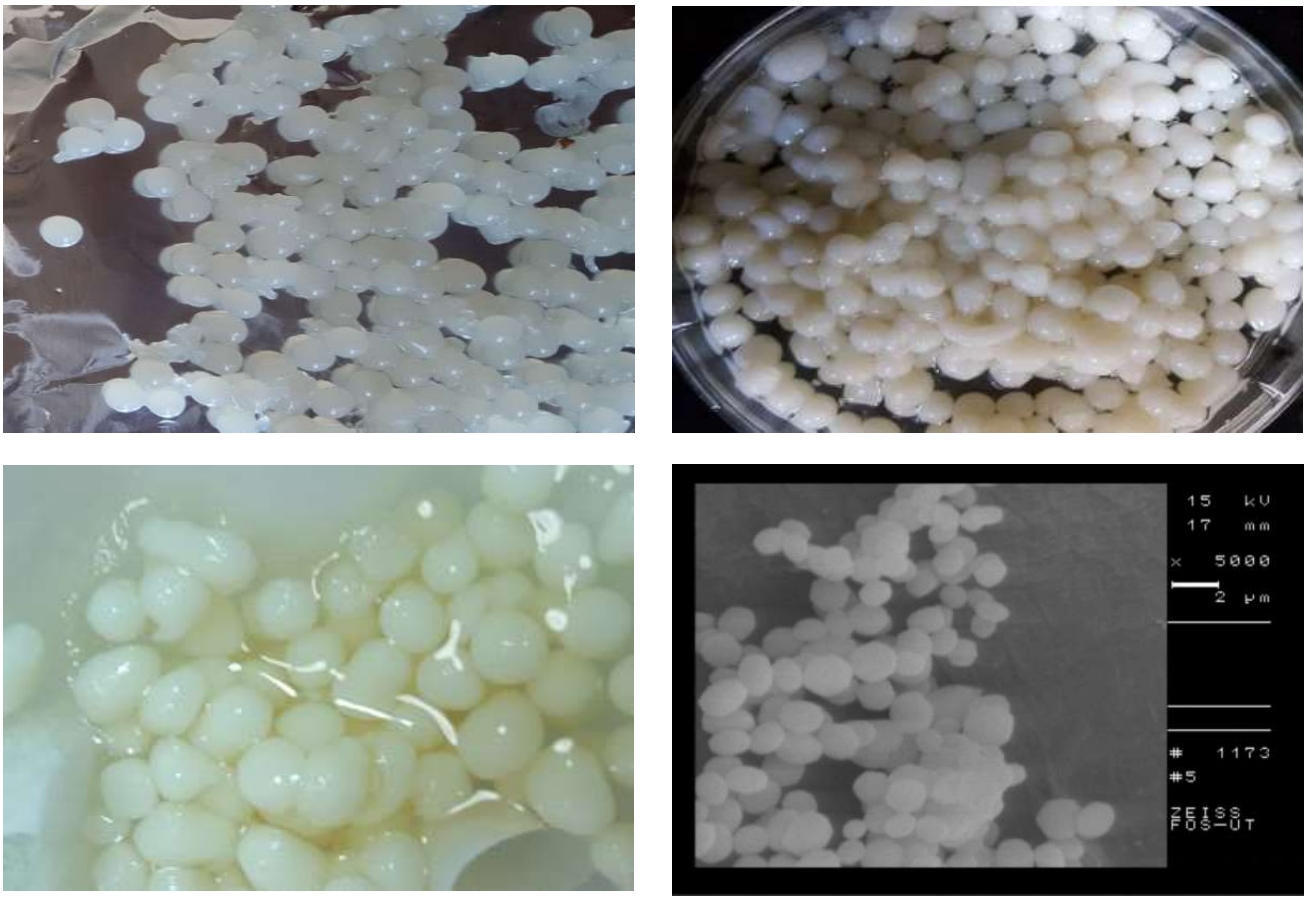
bacteria retention in the polymer was estimated (Arslon-Tontul and Erbas 2017).

Configure of simulated gastric fluid (SGF): An acidic medium (SGF) prepared for the purpose of studying the enzyme and the PH effects (Subirade et al 2003). 1 g of encapsulated and coated beads was transferred to the test tubes containing 9 ml of SGF solution, the tubes were incubated at a temperature of 37°C for (0, one hour, two hours). The same steps described above were followed in for the free cells by transferring 1 ml and followed the method described by (Sabnis and Malavkar 2016).

Configure simulated intestinal fluid (SIF): For calculating the encapsulated and coated beads releasing and to study the enzyme and the pH effects, the method described by (Subirade et al 2003) was used. The same steps described above were conducted for the purpose of studying the effect of pH and the swelling ratio of encapsulated beads and their types was done according to the method of (Ayama et al 2014).

RESULTS AND DISCUSSION

Encapsulation yield (EY): The statistical analysis results showed that there was a significant difference in the yield of binding (Table 1 and Fig. 1). The binding process of encapsulated uncoating layer beads recorded the highest percentage of 95.74%, while the single single-layer beads were characterized by a yield of 91.05%, followed by the encapsulated and coated with double layer coating beads. The decrease in binding the binding may be due to the mechanical processes during the coating and interactions between the layers which contribute to reduction the bio probiotic in the encapsulated beads with single layer coating compared to the double layer, while increasing yield of binding process efficiency of the encapsulated and uncoating beads is due to the bio probiotic adhesion on the surface of the beads. The layer-by-layer coating process was characterized by improving the survival rate of bacteria, increasing the beads diameter, reducing porosity of the polymer and increasing viscosity. These qualities contribute



A. Single layer coating of beads with alginate
B. Double layer coating of beads with chitosan
C. Microstructure under the scanning electron scan microscope (SEM)

Fig. 1. Uncoating beads of encapsulation suspended cell with whey protein

Table 1. Yield of a binding process for the encapsulation and coated bacteria by three types

Coating type	Bacteria numbers before adding	Bacteria numbers after microencapsulation	Yield of binding process (%)
Free cells	9.17	9.17	100
Uncoating	9.17	8.78	95.74
Single layer	9.17	8.35	91.05
Double layer	9.17	8.23	89.74
LSD (p=0.05): 1.48			RLSD: 2.23

to provide more protection to the retain and increase the survival rate of the bio probiotic when exposed to harsh conditions from low pH and pepsin enzyme and bile salts and this increase the efficiency of the binding process. Packaging technology enhances and ensures that the numbers of bacteria reach to intestines and protect them from internal conditions. The reason for the variation in retention the bacteria are due to the type coating, the nature of the charge owned by the material which producing coating and the physical and chemical properties of the polymer used in the micro-packaging process (Ouled-Haddar et al 2016). The encapsulated beads with starch and coated beads with a single layer of alginate does not give a good protection to the bacteria because of rapid its decomposition during its exposed to a low pH, while in the case of encapsulated and coated beads with a double layer of alginate, it improves the coated beads stability, and this is due to that the coating provides better protection for the bio probiotic, and the Chitosan layer was characterized as a thicker and high molecular weight in addition to increasing its size, diameter and reducing its porosity (Ivanovska et al 2012).

Chen et al (2017) explained that there are factors that contribute to improve the survival rate of bacteria in the microencapsulation, which is represented by the spherical shape, diameter and type of coating materials, where the uncoating beads (isolated whey proteins) were characterized by having a diameter of 81.8μm and the binding efficiency reached 96.35%, while the coated beads with a single layer of alginate had a diameter of 118.1 μm. The efficiency of the binding process reached 95.28%. Although decreasing the binding efficiency of single-layer coating beads, but they characterized by a strong gel that made it insoluble, which led to having the resistant trait towards enzymatic decomposition and its resistance to bile salts. This trait contributes to provide more protection in the retention and increased the survival rate of bacteria. The uncoating bead is characterized by the decomposition ability in pepsin enzyme within 30 minutes due to that the whey proteins characterized by porosity; coating helps to reduce porosity and resistance the SGF and SIF. The results were agreed with findings of Ayama et al (2014), Pitigraisorn et el (2017). Pitigraisorn et al (2017) observed the efficiency of the binding process for *L. acidophilus* TISTR 1338 was 85.0-95.3%.

Configure Simulated Gastric Fluid (SGF)

Stability of encapsulated and coated beads: *Lactobacillus casei* bacteria coated with double-layer coating showed the highest stability towards the enzyme with a survival rate of 94%, while in the single-layer coating, cell was 91%, which is higher than that of encapsulated uncoating cells with was 85% (Fig. 2). The free cells recorded the lowest survival rate of 68.15% and the highest reduction rate of 2.92%. This may be due to the nature of polymer material, binding type and the acidic function that made the gel more resistant to enzymatic decomposition and the concentration of the solidification solution that contributes to make the polymer insoluble or resistant to decomposition. In addition, coating with layer by layer earns the encapsulated and coated beads stability towards enzyme and provides better protection to the bio probiotic. This is due to increases in the diameter of coated beads and the interactions between cells and SGF solution, making them more resistant to decomposition. González-Cuello et al (2017) pointed out that coating method with layers of the encapsulated beads had a positive effect in terms of reducing porosity and increasing beads diameter. These physical properties help to provide better protection to the bio probiotic, which improves its survival rate compared to the free bio probiotic. Shu et al (2018) observed the reduction rate of free cells amounted to 3.38, while the reduction rate of single layer cells (xanthan-chitosan) was 2.7, and the double layer cells (xanthan-chitosan-xanthan) reached 1.8. Argin-Soysal et al (2009) and Arslon-Tontul and Erbas (2017) also observed similar trend.

Stability of coated beads towards the acidic medium: There was a decrease in the viable counts log (cfu/ml) for both free and coated cells at pH 2 and the coated beads with double layer coating recorded the lowest reduction rate of 0.77, and the highest survival rate of 90% (Fig. 3). The coated beads with single layer coating had a reduction rate of 1.3% and the survival rate was 84%. The coated beads with double layer coating and single layer coating showed the highest resistance compared to the encapsulated beads with a survival rate of 76.4%, and free cells with a survival rate of 64.5%, and a highest reduction rate of 3.25. This is due to the nature of their binding with Chitosan then alginate and whey proteins, which led forming a polymer with a low porosity and thick layer. This made the beads resist the acidic conditions, enzymes and bile salts. The results were consistent with earlier workers (Annan et al 2008, Mokarram et al 2009, Shi et al 2013) under the pH effect.

Configure simulated intestinal fluid (SIF): There was a significant difference in the cells release percentage for both free and encapsulated cells at the PH 7, where the encapsulated beads recorded 45% at 30 minutes, and the coated beads with double layers coating recorded 14% (Fig. 4). The single-layer coating beads recorded 25% in 30 minutes. The double layer and single layer coating beads showed a highest decomposition resistance compared to the encapsulated uncoating beads due to the nature of their binding with Chitosan then alginate and whey proteins, which led to the formation a polymer that had a low-porosity with a thick layer, this made the beads resist the acidic conditions, enzymes and bile salts. The results were consistent with Pan et al (2013) findings, where the encapsulated cells release percentage of *Lactobacillus bulgaricus* when exposed to SIF under pH effect 6.8 and incubation temperature of 37°C.

Swelling ratio of encapsulated and coated beads: There was a significant difference in the beads swelling ratio, and the double layer coating beads had the highest stability towards enzyme, and the lowest swelling ratio during exposure to pepsin was 1 at 120 minutes, and 3 during exposure to pancreatic at 240 minutes, respectively (Fig. 5). The coated beads with single layer coating had a 1.4 and 3.38 swelling ratio, which is lower than that of encapsulated uncoating beads when exposed to pepsin at 120 minutes and pancreatic at 240 minutes, respectively, which was recorded the highest swelling ratio of 1.6 and 3.55 at 120 minutes under the pepsin effect, and at 240 minutes under pancreatic effect, respectively. The beads are sensitive to the neutral medium, and results in an increase gel swelling ratio and may be attributed to most negative charges were on the groups (Maltais et al 2009). In addition, the rapid polymer decomposition and the survival rate of bio probiotic may be

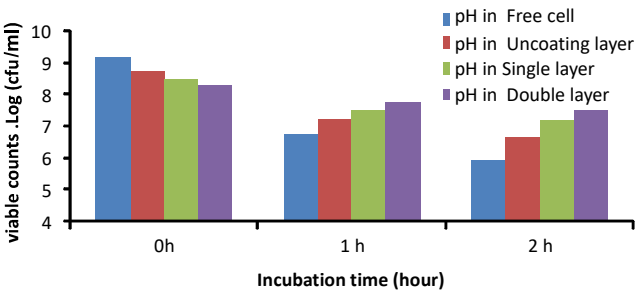
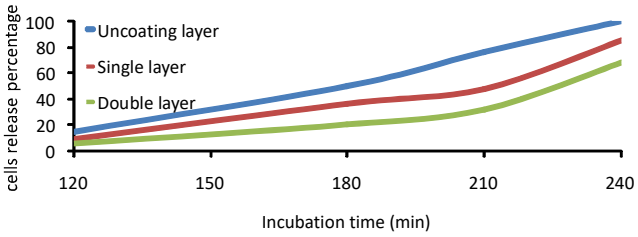


Fig. 3. Stability beads encapsulated and coated beads with *L. casei* of when exposed to SGF under the pH effect



RLSD: 1.173 for types coating
RLSD: 1.73 for time

Fig. 4. Release profile of encapsulated cells in pH 7 in SIF under the enzyme effect

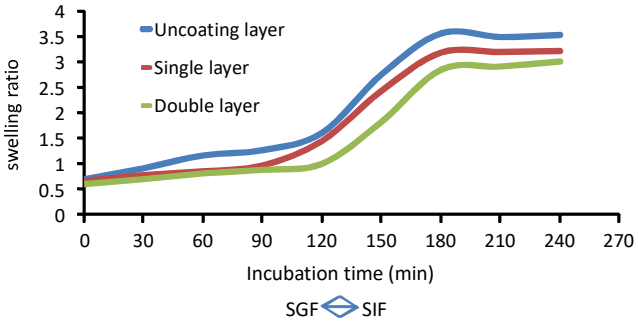


Fig. 5. Swelling rate of encapsulated and coated beads with the presence of enzymes when exposed to SGF

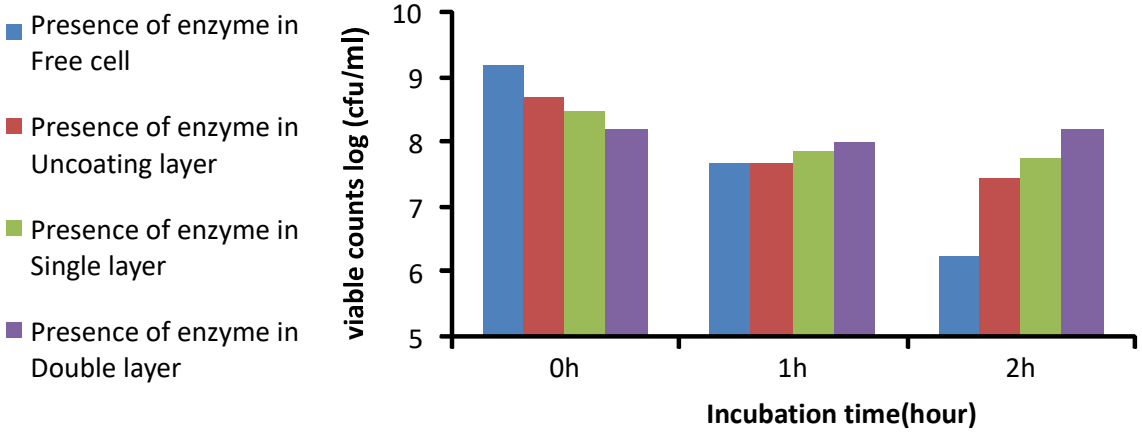


Fig. 2. Stability beads encapsulated and coated beads with *Lactobacillus casei* of when exposed to SGF under the enzyme effect at a different periods time (0, 1 and 2) hour

attributed to the nature of the polymer composition, beads size and diameter, type of acidic function, enzyme type, concentration of bile salts and the bio probiotic strain type, all are contributing to increase or decrease the survival rate of bacteria by Zhang et al (2016) and Wang et al (2014). Earlier studies have shown that the coated beads with single layer and double layer coating are more resistant to enzymatic decomposition when exposed to SIF compared to the encapsulated beads. This may be due to increase the diameter of coated beads making them more stable from the encapsulated beads. The encapsulated uncoating beads showed accelerated the release percentage, and may be due to the rapid the enzymatic decomposition, which accelerates the release of cells and this depends on several factors, including the material used in the decomposed and breakdown the polymer and the neutral function which leads to increase the swelling ratio of beads and this contribute to release (Chen et al 2017) evaluated the efficiency of binding process and the use of the modern coating method by layer by layer. And reported that survival rate of the cells decreases in a single layer coating and reached 54, while in a double layer coating was 64 when the beads were exposed to the SIF solution with the presence of enzyme.

The swelling rate during exposed to (SGF) under the acidic function effect, as the encapsulated uncoating beads was recorded the highest swelling rate reached 1.71, and during exposed to (SIF) under the neutral medium effect it was recorded the highest swelling rate amounted to 3.8, while the encapsulated and coated beads with single layer coating showed the lowest swelling rate reached 1.45 during exposed to (SGF) at 120 minutes (Fig. 6, 7). The lowest swelling rate during exposed to SIF was under the neutral medium effect of 3.58 at 240 minutes, whereas the coated beads with double layer coating had the lowest swelling rate of 1.3. Similarly when exposed to (SIF under the neutral medium reached 3.36 at 240 minutes. The uncoating beads had a rapid decomposition and swelling compared with the rest of the coating beads and this enhances the reduction of bacteria cell numbers in these beads and in both cases under the enzymatic or the pH effect as shown in the figure below.

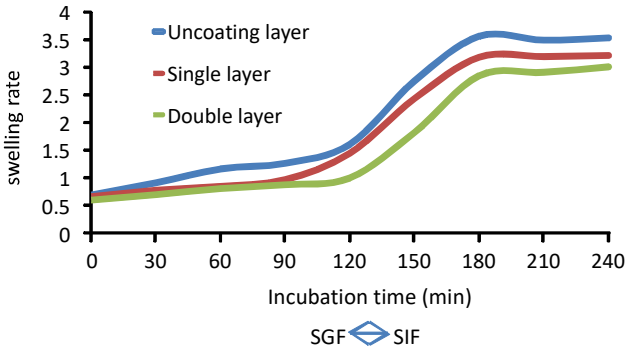


Fig. 6. Swelling rate of encapsulated and coated beads under the acidic and neutral medium effect while the swelling rate of encapsulated and coated beads during exposure to SIF

Fig. 7. A: encapsulated beads submerged in solution SGF B: encapsulated beads submerged in solution SIF C: after 60 min submerged in solution SIF

CONCLUSIONS

The nature of the coating material plays an important role in maintaining the bio probiotic numbers and this depends on coating type which have a significant impact on protect of probiotic before the increase in the efficiency of encapsulated yield. The highest efficiency of coating of the enhanced uncoated layer 95.74%, while the single layer coated reached 91.05 and % double layer coated 89.74 during its exposed to harsh conditions represented of enzymes and acidic function during a different periods time The cell release of encapsulated and coated with its three types in SIF at 30 minutes enhanced double layer coated. The double layer coated decrease, while encapsulated uncoated resulted in higher swelling. The coated beads with double layer coating recorded the lowest swelling rate and highest stability towards two types of enzymes, which provided the best protection for the bio probiotic.

ACKNOWLEDGMENTS

The authors acknowledge the support from the University of Basrah, college of Agriculture, Department of Food Science. The authors thank angel Department of food and Technology, college of Agriculture, Department of Science assistance in the Statistical analysis.

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Received 13 January, 2020; Accepted 31 May, 2020